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Effects of specific antisera targeting peritrophic matrix-associated proteins in the sand fly vector *Phlebotomus papatasi*

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ABSTRACT

In many hematophagous insects, the peritrophic matrix (PM) is formed soon after a blood meal (PBM) to compartmentalize the food bolus. The PM is an important component of vector competence, functioning as a barrier to the development of many pathogens including parasites of the genus *Leishmania* transmitted by sand flies. PM morphology and permeability are associated with the proteins that are part of the PM scaffolding, including several peritrophins, and chitin fibers. Here, we assessed the effects of specific antisera targeting proteins thought to be an integral part of the PM scaffolding and its process of maturation and degradation. *Phlebotomus papatasi* sand flies were fed with red blood cells reconstituted with antisera targeting the chitinase PpChit1, and the peritrophin PpPer2. Sand fly midguts were dissected at different time points and processed for light microscopy (LM), confocal and transmission electron (TEM) microscopies (24, 42–46, 48 and 72 h PBM), scanning electron (SEM) (48 h PBM) and atomic force (AFM) (30 h PBM) microscopies. TEM and WGA-FITC staining indicate PM degradation was significantly delayed following feeding of flies on anti-PpChit1. AFM analysis at 30 h PBM point to an increase in roughness' amplitude of the PM of flies that fed on either anti-PpChit1 or anti-PpPer2. Collective, our data suggest that antibodies targeting PM-associated proteins affects the kinetics of PM maturation, delaying its degradation and disruption and are potential targets on transmission-blocking vaccines strategies.

1. Introduction

The peritrophic matrix (PM) is a non-cellular semi-permeable layer composed of chitin fibrils associated with glycoproteins and secreted in the midgut of most of insects. It forms an envelope surrounding food bolus in the midgut lumen separating luminal contents into two functional compartments, the endo and the ectoperitrophic spaces (Lehane and Billingsley, 1996). The PM is characterized as either type I when it is secreted by digestive cells in response to the epithelium distention that occurs following a blood meal or type 2 when secreted continuously by specialized cells present in the cardia (Peters, 1992; Lehane, 1997; Shao et al., 2001; Devenport and Jacobs-Lorena, 2005). The PM plays a key role associated with the efficiency of digestion and absorption of nutrients, as its permeability and porosity allow for a selective movement of molecules, such as digestive enzymes, from the gut lumen into endoperitrophic space (Lehane and Billingsley, 1996; Hegedus et al., 2009). The PM also functions as a limiting barrier to microorganisms, including many pathogens, and protects the microvilli from abrasion during digestion (Tellam et al., 1999; Lehane, 1997).

Phlebotomine sand flies are vectors of leishmaniasis, a multispectrum disease ranging from mild cutaneous to an often-fatal visceral form caused by parasites of the genus *Leishmania*. Such parasites display a complex developmental cycle within the sand fly vector that is limited to the midgut of the insect. Infection of the sand fly occurs when the parasites present primarily in macrophages and other cells of the mononuclear phagocyte system are picked up during a blood meal on an infected vertebrate. However, for subsequent transmission certain steps and criteria typically associated with the *Leishmania* ability to multiply and undergo metacyclogenesis in the vector must be met. Among such steps are the capacity of parasites to survive a proteolytic attack in the gut, escape from the endoperitrophic space presumably by

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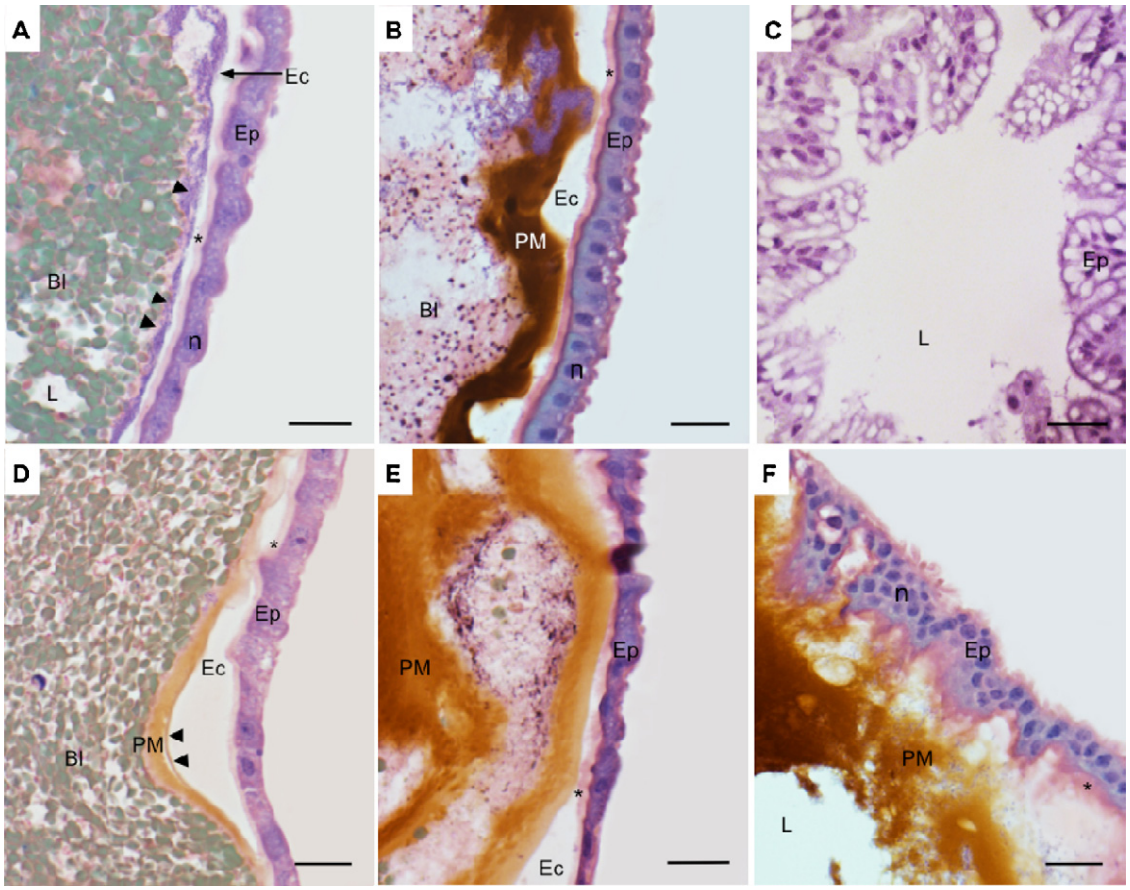

Fig. 1. Histological sections of midgut of *P. papatasi* (PPIS), stained with hematoxylin and eosin (HE). Insects fed with RBC reconstituted with naïve sera (control) (A–C) or fed with RBC reconstituted with anti-PpChit1 (D–F). The midguts were dissected 24 h (A and D), 48 h (B and E) and 72 h (C and F) PBM, respectively. Midgut epithelium (Ep) with digestive cell nuclei (n) and brush border (*) is shown. The blood bolus (Bl) containing the blood cells is seen at 24 h and 48 h PBM. The peritrophic matrix (PM, and black arrows) is thin and thick 24 h and 48 h PBM, respectively. Note that the PM is still thick in the treatments with anti-PpChit1 72 h PBM. Ectoperitrophic space (Ec). Lumen (L). Bar = 10 μm.

crossing the PM, and bind to the midgut epithelium, preventing excretion (Bates and Rogers, 2004; Ramalho-Ortigao, 2010). Hence, in sand flies, the PM is an important component of the vector competence, (Sacks and Kamhawi, 2001; Coutinho-Abreu et al., 2010).

It has been shown that in sand flies, the speed in which the PM components are secreted (Secundino et al., 2005; Pruzinova et al., 2015) and the PM kinetics (Sádlová and Volf, 2009; Pruzinova et al., 2015) can vary according to species. However, it is generally accepted that a balance between synthesis and degradation, presumably through the actions of chitin synthases and chitinases, determines PM thickness (Shao et al., 2001). It has also been shown that a thicker PM, produced by the addition of chitinase inhibitors to the blood meal, can trap of *Leishmania* within the endoperitrophic space (Pimenta et al., 1997).

We previously characterized the midgut chitinases LlChit1 and PpChit1 from the sand flies *L. longipalpis* (Ramalho-Ortigão and Traub-Csekö, 2003) and *P. papatasi*, respectively, and showed that PpChit1 is involved in the formation and degradation of the PM (Ramalho-Ortigão et al., 2005; Coutinho-Abreu et al., 2010). Further, out of three peritrophins we also identified in *P. papatasi*, two were shown to be involved in the formation and the scaffolding of the PM in sand flies (Ramalho-Ortigão et al., 2007; Coutinho-Abreu et al., 2013). Peritrophins bind to chitin via their cysteine-rich chitin biding domains (CBD), and have a crucial role determining the elasticity and permeability of the PM (Lehane, 1997; Shao et al., 2005).

Here, we investigated aspects of PM structure and kinetics in the sand fly *P. papatasi* following feeding on blood (RBCs) containing antisera targeting the proteins PpChit1 and PpPer2. PM morphology was assessed by means several microscopy techniques, including TEM, confocal, and atomic force. Our results indicate that feeding the specific antisera anti-PpChit1 or anti-PpPer2 led to changes of the PM structure and kinetics, specifically with regards to its overall thickness and degradation kinetics. Our data provides further support to transmission blocking approaches to prevent *Leishmania* transmission by sand flies by targeting molecules involved in the formation and degradation of the sand fly PM.

2. Materials and methods

2.1. Mice

BALB/c mice, 8–12 weeks in age, were raised and maintained under pathogen-free conditions at the Kansas State University animal facility. The use of animals during this study was reviewed and approved by the Kansas State University IACUC under protocol number #4484.

2.2. Antisera production

Specific antisera to PpChit1 and PpPer2 were produced and purified as previously described (Coutinho-Abreu et al., 2010). Plasmids encoding each mature protein were purified and then sterilized by filtration using a 0.2-μm filter (Millipore). A volume of 10 μl (10 μg/μl) of the purified plasmid was injected subcutaneously into the ears of isoflurane-anesthetised female BALB/c mice 8–12

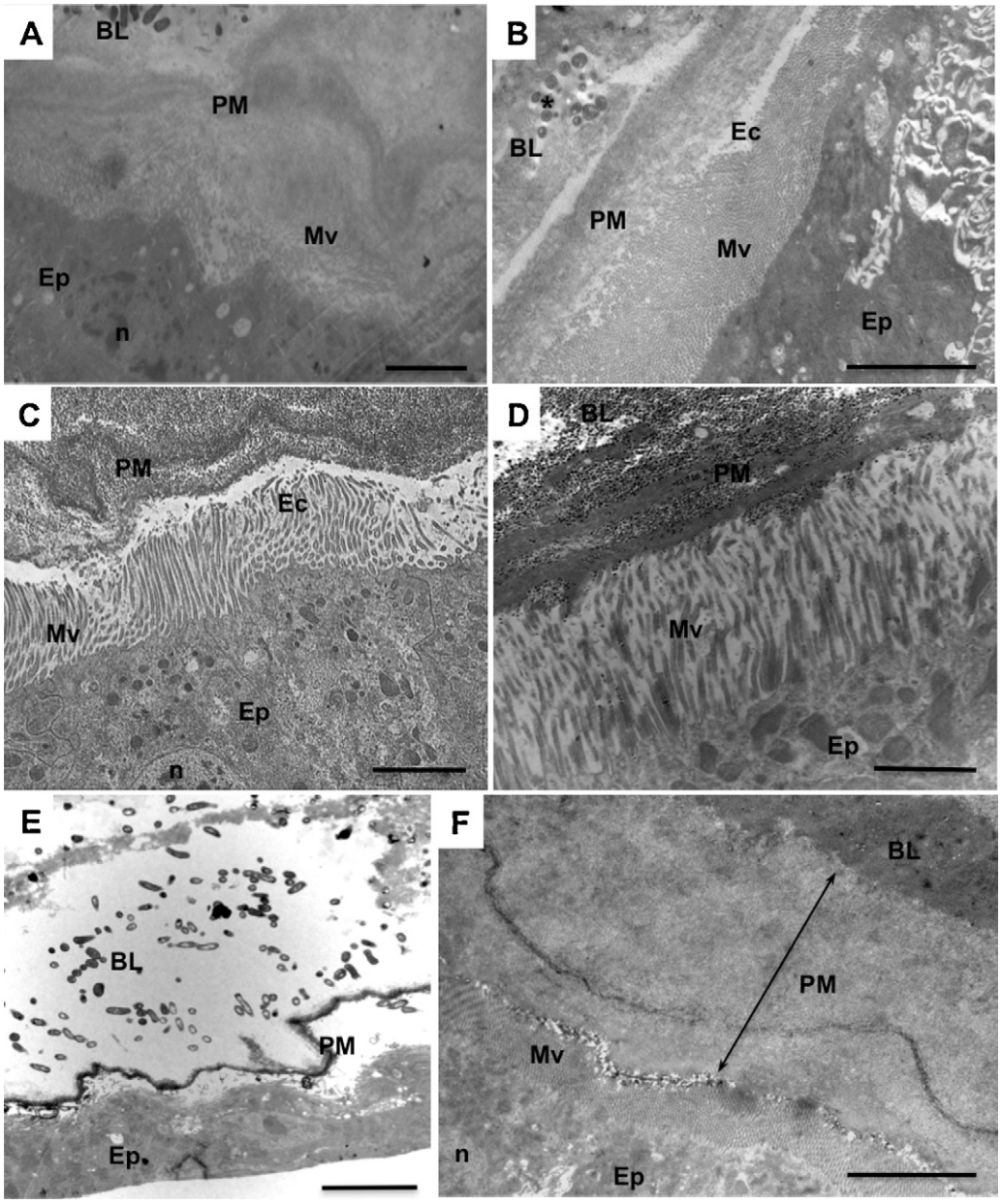

Fig. 2. Transmission electron micrographs of the midgut of *P. papatasi*. Sand flies fed with RBC reconstituted with naïve sera (A, C, E) or with RBC reconstituted with anti-PpChit1 (B, D, F). The midguts were dissected 24, 48 and 72 h PBM. Epithelium (Ep). Ectoperitrophic space (Ec). Peritrophic Matrix (PM). Microvilli (Mv). Nuclei of the digestive cells (n). Food bolus (BL). PM thickness (black arrows) (F). Bars = 5 μm (A, B, E and F); 9 μm (C); 2 μm (D).

weeks of age, using a 29.5 gauge needle. A total of three injections per mouse ear were done, each two weeks apart. Two weeks after the last DNA immunization, about 300 ml of blood was collected from the sub-mandibular vein from each mouse. Collected serum was separated by centrifugation at 750 rpm for 10 min (Eppendorf, USA). The titer of antibody obtained was measured with the Easy-Titer Kit IgG Assay Kit (Pierce, Rockford, IL) following the manufacturer's recommendations and kept at −20 °C until use.

2.3. Rearing and feeding of sand flies

Phlebotomus papatasi Israeli strain (PPIS) were reared in the Department of Entomology, Kansas State University, under 12 h by 12 h (12 h:12 h) light/dark cycles and at 26 °C and 80% RH. Two groups, one experimental and one control, with roughly 150 females (3–5 days old) each were used. Each experiment was conducted separately. Red blood cells (RBC) were collected by centrifugation from blood obtained from a naïve (non immunized) mouse and reconstituted with 195 μl of serum naive (control) or with 195 μl of specific antisera, either anti-PpChit1 or anti-PpPer2. Sand flies were allowed to feed for 1 h using chicken skin membranes placed on glass feeders attached to a circulating water bath set at 37 °C.

2.4. Morphology

2.4.1. Dissection

Adult *P. papatasi* females were anesthetized with CO_2, washed briefly in soapy water and transferred ice cold 0.1 M PBS immediately prior to dissection. Dissections were done under a Zeiss 2000 stereoscope. Midguts were dissected in 0.1 M PBS and placed in fixative solution (either 0.25% glutaraldehyde, 2.5% glutaraldehyde or

4% paraformaldehyde) and maintained at 4 °C for at least 2 h until use.

2.4.2. Light microscopy

For histological analysis, five midguts per time point were dissected from flies fed on anti-PpChit1 sera at 24 h, 48 h and 72 h PBM, and transferred to a fixative solution (glutaraldehyde 2.5%, 0.1 M sodium cacodylate buffer pH 7.4) for at least 2 h. Guts were washed 2X in PBS and dehydrated in graded ethanol series for 5 min each bath (30–100%), infiltrated in Ethanol/historesin Leica® (Leica, Nussloch, Germany) (1:1) for 10 min and historesin without hardener for at least 1 h. Samples were embedded in historesin and hardener solution at room temperature according to the manufacturer's protocol. Slices 3 μm-thick obtained using glass blades and microtome were stained with hematoxylin and eosin (HE). The dried slides were mounted in Eukitt® mounting medium (Sigma-Aldrich, St. Louis, USA) and photographed under an optical microscope Olympus BX 53 coupled with an Olympus DP73 digital camera.

2.4.3. Transmission electron microscopy (TEM)

Eight midguts each fed either with anti-PpChit1, anti-PpPer2, or naive sera were dissected in PBS, transferred to fixative (2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer pH 7.4) and held for 2 h at room temperature to the subsequent steps. Guts were washed in cacodylate buffer and post-fixed for 2 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Following five washes in PBS guts were dehydrated in graded ethanol series (30–100%), preinfiltrated in LRWhite® acrylic resin (London Resin Company Ltd., England) and 100% ethanol 2:1, 1:1, and embedded in 100% resin overnight. The inclusion of resin was carried out at 60 °C in gelatin capsules for 48 h. Ultrathin sections (70–90 nm) were contrasted with 2% aqueous uranyl acetate and 1% lead citrate and analyzed using a Zeiss EM 109 transmission electron microscope.

2.4.4. Atomic force microscopy (AFM)

Five PMs were dissected intact, 30 h PBM, from females treated with anti-PpChit1, anti-PpPer2 and control in 50% ethanol in PBS 1% and fixed in 0.25% glutaraldehyde. Dissection of PMs from flies treated with anti-PpPer2 at times later than 30 h PBM proved to be extremely difficult, as the PM were highly degraded and completely falling apart during the dissection. PMs were dehydrated in graded ethanol series (30–100%) and dried HMDS. The inner surface (corresponding to the endoperitrophic space) was positioned under a cover slip of 1 cm^2 and evaluated in semi-contact mode in a scanning probe microscope model (Integrates Probe Nanolaboratory Molecular Devices and Tools mode Nanotechnology) (NT-MDT, Moscow, Russia).

2.4.5. Laser confocal microscopy/WGA-FITC labeling

To assess for the presence of polysaccharides containing β-1-4 *N*-acetyl-glucosamine residues, sand flies were fed with anti-PpChit1, anti-PpPer2, and naïve sera and guts dissected at 48 h and 72 h PBM. Six midguts from each group were dissected and fixed in Zamboni's solution (picric acid and formaldehyde), washed 3× for 30 min each in PBS containing 1% Tween (PBST), incubated 1 h at room temperature with FITC-conjugated wheat germ agglutinin (WGA) (Sigma-Aldrich, #L4895, Israel) diluted 1:1000 in 0.1 M PBS. Guts were washed 3 times in PBS for 5 min each and the cells' nuclei were labeled with TO-PRO-3 (Thermo Fisher Scientific, Waltham, MA USA) diluted 1:1000 in 0.1 M PBS for 1 h. Following a final set of three 5 min washes in PBS, slides were mounted with Mowiol solution and analyzed under confocal microscope Zeiss 510 LSM NMM/UFV. Fluorescence intensity was quantified using the Zeiss LSM Image Examiner (version 4.0.0).

2.4.6. Statistical analyses

Statistical analyses were performed using Statistica 7.0 software (Copyright Statsoft Inc, USA). For all treatments, mean and standard deviation were calculated and considered significant at 5% ($P < 0.05$). Initially, data distribution was assessed for normality by the Shapiro-Wilks test. However, due to its non-normal distribution, differences between treatments were assessed using the nonparametric Kruskal-Wallis test and Will Cox-Mann-Whitney.

3. Results

3.1. Histology

The midgut epithelium in *P. papatasi* is formed by a typical single layer of columnar cells with central nuclei. These cells are supported by a basal lamina and display a well-developed brush border on the apical portion (Fig. 1). Following the blood meal, at 24 h PBM, the secreted PM appeared to be similar between sand flies fed on RBCs that were reconstituted either in the anti-PpChit1or the control naïve sera, with a thin layer separating the blood bolus just above the midgut epithelium. At 48 h PBM, the PM appeared completely formed in all three groups, and with much thicker and with darker aspect than at 24 h PBM. The dark coloration present throughout the PM (Fig. 1) is likely due to heme that can be bound to heme-binding motifs present in *P. papatasi* peritrophins (Coutinho-Abreu et al., 2013). Interestingly, differences in the PM were noted at 72 h PBM between flies that fed on either specific antisera anti-PpChit1 or flies that fed on the naïve serum. The PM of the individuals fed on either antisera was still very much preserved and considerably ticker that in the control sera fed flies, and suggestive of a delay in the degradation of the PM in the specific antisera fed flies. In addition, traces of blood and blood cells were still visible within the food bolus of such flies (Figs. 1 and 2). In stark contrast, the PM of flies that fed on the naïve sera significantly degraded (Fig. 1A–C).

3.2. Transmission electron microscopy (TEM)

The TEM confirmed the midgut epithelium cells of *P. papatasi* are supported by a basal lamina, with the apical portion covered with numerous microvilli arranged in parallel (Fig. 2). The overall thickness of the PM at 24 h and 48 h PBM was similar for flies fed either with the specific antisera or fed on the control serum (Fig. 2A–D). Curiously, at 72 h PBM, the anti-PpChit1 fed flies had a thicker PM and greater numbers of blood cells compared to the naïve sera-fed flies (Fig. 2E and F). The PM was also evaluated in sand flies fed with the anti-PpPer2 specific antiserum at 30 h and between 42 and 46 h PBM. Thirty hours after feeding, the general structuring of the epithelia and the PM were similar for both anti-PpPer2 fed and naïve serum fed: for the former, cells rich in mitochondria, and covered with microvilli on the apical portion; and for the latter, the well-structured PMs. However, between 42-to-46 h PBM, sand flies fed with anti-PpPer2 displayed a porous PM in comparison to the naïve sera fed flies (Fig. 3D).

3.3. Atomic force microscopy (AFM)

Next we compared the topography of the PM dissected at 30 h PBM from sand flies fed on anti-PpChit1, anti-PpPer2 and naïve sera reconstituted RBCs using AFM in semi-contact mode. For flies fed on either of the two specific antisera, an increase of the amplitude of the PM surface roughness was observed compared to the PM obtained from the naive sera fed flies. Furthermore, the PM thickness was also significantly altered, with flies that fed on the anti-PpPer2 antisera being twice as thick as flies that fed on naive

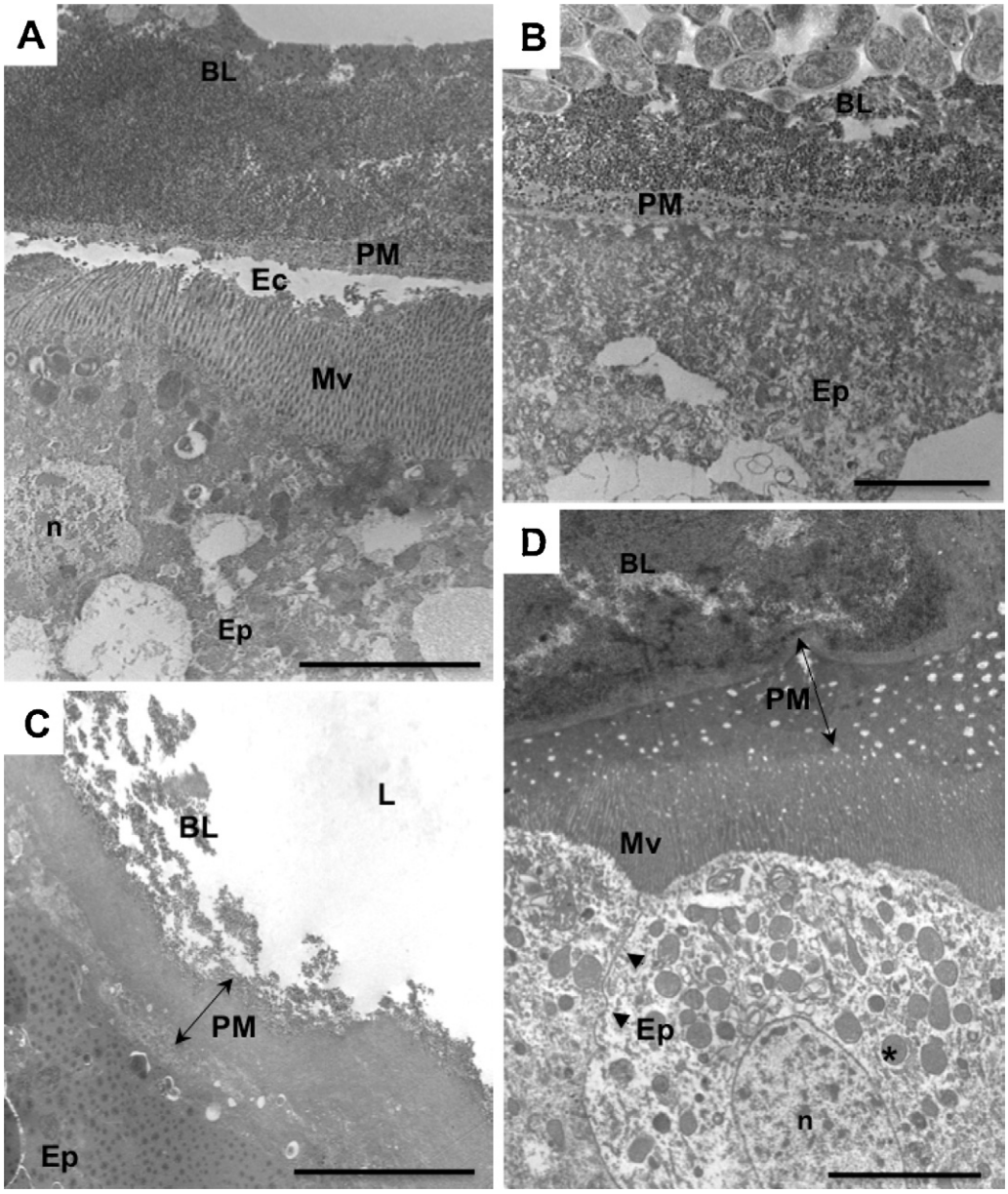

Fig. 3. Transmission electron micrographs of the midgut of *P. papatasi*. Sand flies fed with RBC reconstituted with naïve sera (A and C) or with RBC reconstituted with anti-PpPer2 sera (B and D). The midguts were dissected at 30 h PBM (A and B) or between 42 and 46 h PBM (C and D). Cell membrane (arrowheads). Epithelium (Ep). Ectoperitrophic space (Ec). Peritrophic matrix (PM). PM thickness (black arrows) (C–D). Microvilli (Mv). Nuclei of the digestive cells (n). Mitochondria (*). Food bolus (BL). Lumen (L). Bar = 5 μm (A, C and D). Bar = 2 μm (B).

Table 1
PM internal face roughness for each area analyzed. Mean and standard deviation (sd) are also shown for each area measured.

Treatment	Amplitude			
	Area 50 μm^2	Area 20 μm^2	Area 16 μm^2	Area 10 μm^2
Control–naïve sera	230 nm (mean 242.5; sd 64.1)	310 nm (mean 225; sd 53.9)	175 nm (mean 160; sd 39.7)	105 nm (mean 135; sd 42.4)
Anti-PpChit1	650 nm (mean 675; sd 80.4)	600 nm (mean 570; sd 40.4)	400 nm (mean 225; sd 112.5)	550 nm (mean 370; sd 105.4)
Anti-PpPer2	450 nm (mean 420; sd 42.4)	325 nm (mean 367.5; sd 60.1)	300 nm (mean 440; sd 198)	260 nm (mean 250; sd 14.1)

sera, and the flies that fed on anti-PpChit1 displaying a PM approximately 3-fold as thick as the control flies (Table 1 and Fig. 4). The amplitude was measured using the roughness profile of each sample, by subtracting the lowest point of the profile from the highest point. To our knowledge this is the first time AFM is used to define the effects of antisera targeting insect PM, or for that matter any proteinaceous structure.

3.4. FITC-conjugated WGA labeling of sand fly midguts (confocal microscopy)

Finally, we assessed the overall amount of chitin present in the PM of sand flies, according to the different feeding regimens in this study, by using FITC-WGA. FITC-WGA binds to the *N*-acetyl-glucosamine (NAG) residues. Analysis of the images and the intensity of fluorescence signal indicate that, by 72 h PBM, the PM

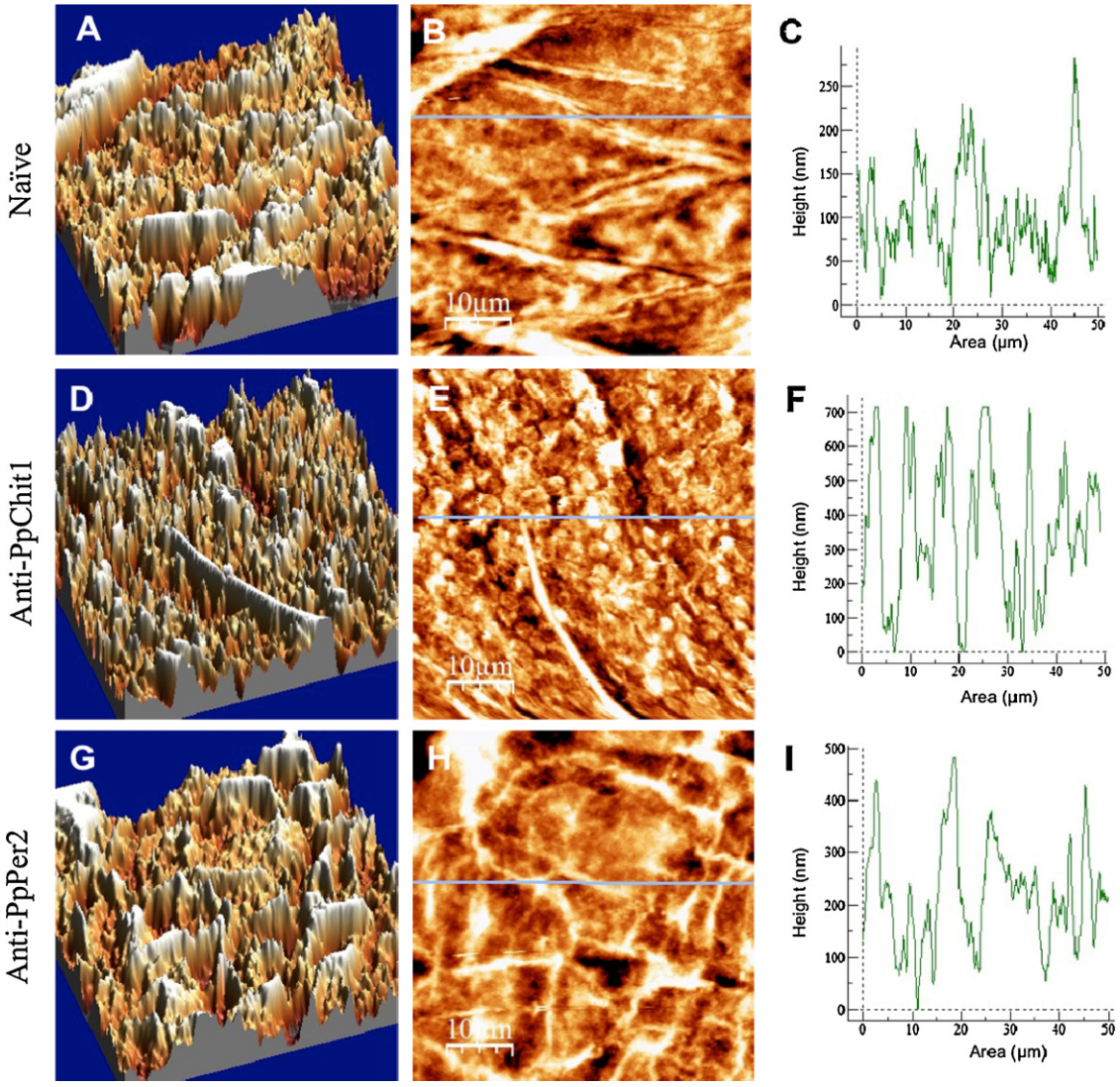

Fig. 4. Atomic force microscopy showing the topography of the inner face of *P. papatasi* peritrophic matrix 30 h PBM. A, B and C correspond to the control. In D, E and F sand flies were fed with blood containing antiserum anti-PpChit1. In G, H and I sand flies were fed with blood containing antiserum anti-PpPer2. The left column represents the 3D image, the middle column represents the digital image of sample topography and the right column represents the amplitude chart of roughness in 50 μm^2 PM area.

of sand flies fed with anti-PpChit1 or anti-PpPer2 were significantly more intensely stained than the naïve sera fed flies ($P < 0.05$) (Figs. 5 and 6). In this study, the FITC-WGA labeling served as a proxy to determine PM stability.

4. Discussion

We investigated the effects of specific antisera targeting the PM-associated proteins PpChit1 and PpPer2 on the kinetics of PM formation and degradation in the sand fly *P. papatasi*. Whereas PpChit1 is a midgut-specific chitinase involved in the maturation and degradation of PM (Ramalho-Ortigão et al., 2005), the peritrophins PpPer1 and PpPer2 likely are integral to the PM scaffolding in *P. papatasi* (Coutinho-Abreu et al., 2013).

We show that feeding sand flies with RBCs reconstituted with antisera targeting PpChit1 and PpPer2 lead to a delay in the degradation of PM in comparison to flies fed on RBCs reconstituted with naïve sera. Such delay in the PM degradation was verified through the persistence and increased thickness of the PM at 72 h PBM compared to controls. A concomitant delay in the excretion of the blood bolus, as shown by presence of blood remains in the gut lumen, also was observed. Further, the comparative microscopy analyses of PMs obtained either by feeding flies on RBCs reconstituted with naïve sera or with the anti-PpChit1 or anti-PpPer2 sera indicated that the feeding using either specific antisera led to structural changes of the PM not observed in the naïve sera-fed flies. Such changes include a significantly thicker PM that is maintained in the anti-PpChit1 fed flies by 72 h PBM, as observed from the TEM, and with significantly alterations to the PM surface, represented by the roughness detected with the AFM.

It is generally accepted that the synthesis of the PM relies on a balance between chitin deposition and chitin (Pimenta et al., 1997). PM degradation is achieved by cleavage of the β-(1–4) glycosidic bonds present in the chitin microfibrils (Merzendorfer, 2003), and catalyzed by midgut chitinases such as PpChit1. It has been shown that addition of an exogenous chitinase in the blood meal leads to lack of PM formation in *P. papatasi* midgut (Pimenta et al., 1997; Araújo, 2012). Conversely, when sand flies were fed with blood containing the chitinase inhibitor allosamidin the PM formed was more dense and thicker (Pimenta et al., 1997). From the AFM analysis and in consonance with these data, the thickening of the PM after feeding anti-PpChit1 may be due to the lack of structural organization caused by the imbalance during chitin deposition without the modulating effect expected of the chitinase PpChit1.

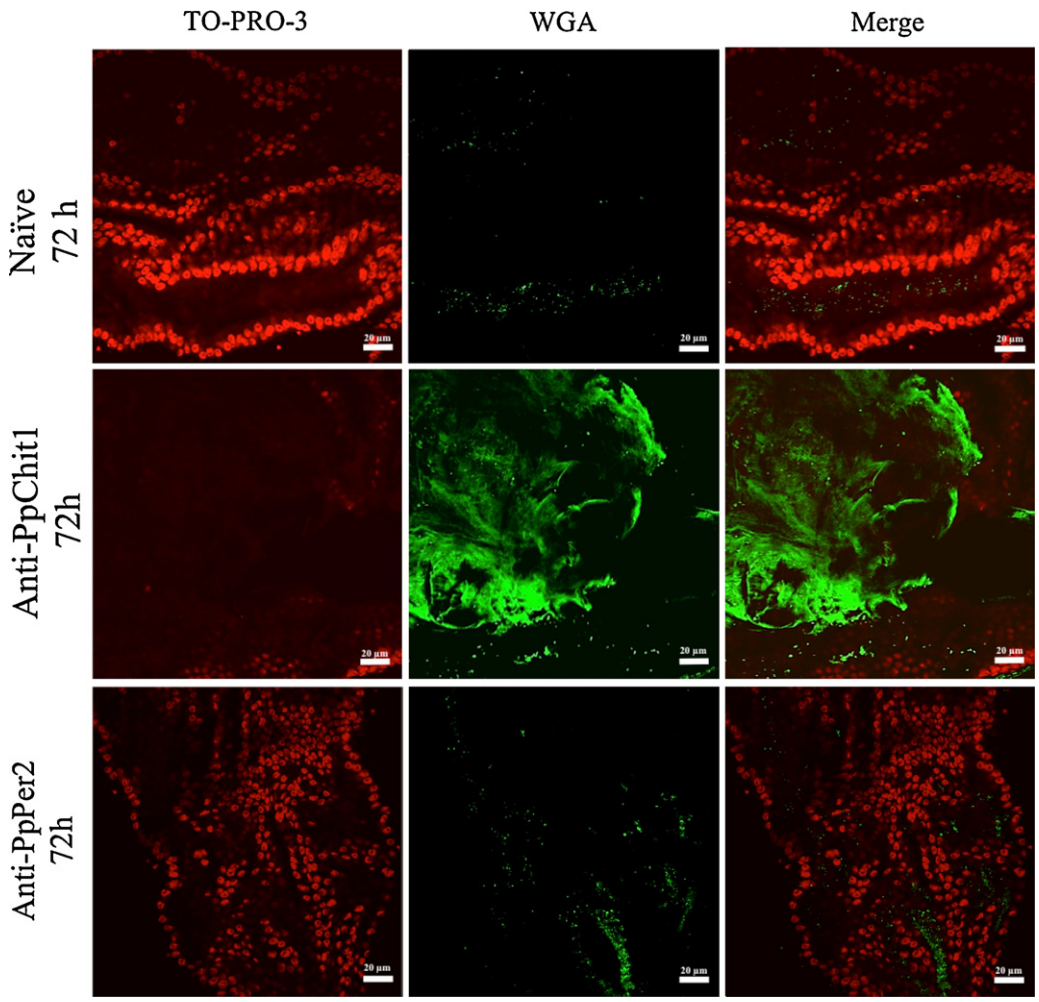

Fig. 5. *P. papatasi* female midgut stained with WGA-FITC (green) and TO-PRO-3 (red). Panels show midguts dissected from sand flies 72 h after feeding on RBC reconstituted with naïve serum, or with anti-PpChit1 or anti-PpPer2 antisera, and hybridized with FITC-labeled WGA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

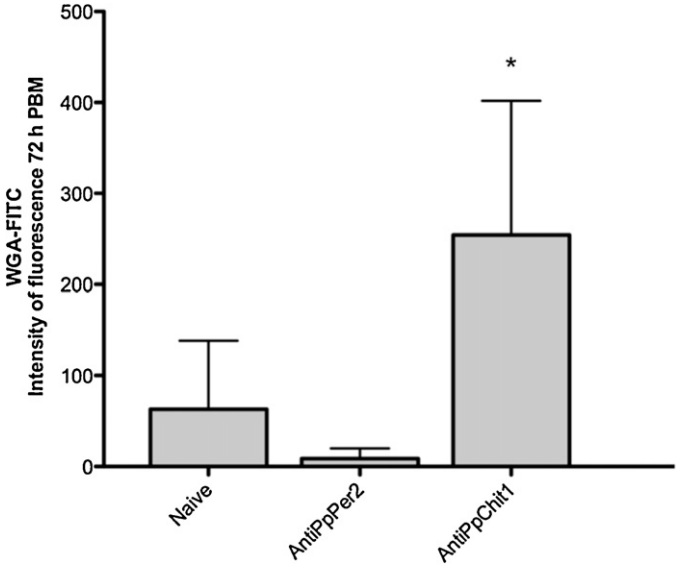

Fig. 6. WGA-FITC fluorescence intensity in the midgut of *P. papatasi*. Mean and standard deviation are shown (bars). A significant difference ($P < 0.05$, Kruskal-Wallis) was observed in the FITC fluorescence intensity flies fed on the naive versus the anti-PpChit1 sera.

The interaction of CBDs present in peritrophins with the glycoproteins that make up a large portion of the components of the PM is critical for the scaffolding that maintains the PM molecular structure and its three-dimensional network (Shao et al., 2005; Ramalho-Ortigão et al., 2007). Feeding sand flies with the anti-PpPer2 sera led to a thicker but at the same time more porous PM compared to the PM in flies fed the control sera. We speculate that such structural disruption of the PM was possibly caused by the disruption of the peritrophin-glycoprotein interactions, either by direct blocking or by allosteric change of the CBDs following binding of the antisera targeting the two peritrophins.

The WGA-FITC labeling points to the presence of significant amounts of PM-associated chitin 72 h PBM in sand flies fed anti-PpChit1. Much less PM-associated chitin is detected in naïve sera-fed flies. The accumulation of chitin in the anti-PpChit1 fed flies potentially is the result of the imbalance between synthesis and degradation, which typically occurs during PM maturation (Shao et al., 2001), caused by the antisera. Whereas chitin is an essential component of the PM scaffolding (Kelkenberg et al., 2015), the chitin accumulation this late in the digestive process evidenced by WGA-FITC binding points to a reduction of the chitinolytic activity in the gut with a corresponding increase in PM thickness and reduction in its permeability.

It has been suggested that a delay in digestion and absorption of nutrients can be caused by changes in the PM (Terra, 2001; Hegedus

et al., 2009). In the sand fly *L. longipalpis*, addition of exogenous chitinase was linked to a faster acquisition of nutrients due to the lack of the PM (Araújo, 2012). Hence, PM thickness and permeability (or porosity) may be associated with effects on digestion and insect fitness (Shen and Jacobs-lorena, 1997). Fitness parameters such as egg laying, rate of excretion, and survival, among others, were investigated previously (Robles-Murguia et al., 2014). Interestingly, flies fed on anti-PpChit1 sera lived longer than flies fed on naïve sera. The anti-PpChit1 sera also had a negative effect on the ability of flies to lay eggs. These effects were linked to the thicker and longer lasting PM, presumably able to scavenge more heme thus reducing its toxicity (Pascoa et al., 2002; Devenport et al., 2006), and reducing the flow of nutrients towards egg development.

We demonstrated that whereas the RNAi knockdown (KD) of PpChit1 significantly reduced *Leismania major* load within the midgut of its natural vector *P. papatasi* (Coutinho-Abreu et al., 2010), PpPer1 KD led to an increase in the parasite load (Coutinho-Abreu et al., 2013). These results highlighted the role played by PpChit1 and PpPer1 during formation, including scaffolding, and degradation of PM, and the PM role as a barrier to *Leishmania*. The data also supported targeting sand fly midgut chitinases, such as PpChit1, in vector based approaches for transmission blocking. The feeding of specific antisera targeting sand fly molecules associated with *Leishmania* development was demonstrated in the seminal study by Kamhawi et al. (2004) targeting the *L. major* LPG receptor in *P. papatasi*. In *P. papatasi*, midgut chitinolytic activity reaches a peak at approximately 48 h PBM, presumably when *Leishmania* escape from the endoperitrophic space is also occurring. We also previously demonstrated that the anti-PpChit1 serum was effective in significantly reducing the activity of recombinant as well as native forms of PpChit1, with a cross-species activity (Ramalho-Ortigão et al., 2005). Although we have yet to demonstrate a direct effect of anti-PpChit1 in the transmission of *Leishmania* by sand flies to naïve vertebrates, for example by trapping parasites within the PM, the thicker and longer lasting PM produced by feeding sand flies anti-PpChit1 is a step towards such goal. As altering aspects of the PM structure can lead to reduction in the load of *Leishmania* within the sand fly vector, we believe that PpChit1, its orthologs, or peritrophins involved in the scaffolding of the PM, can provide meaningful target(s) for transmission reduction. It is also conceivable that, in combination with current strategies such as vaccination of dogs against transmission of *Leishmania infantum* (Nogueira et al., 2005; Saraiva et al., 2006; Borja-Cabrera et al., 2008), transmission elimination for certain *Leishmania* species may be achieved.

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